Glucose Modulates Basement Membrane Fibroblast Growth Factor-2 via Alterations in Endothelial Cell Permeability*

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The effects of glucose extremes on vascular physiology and endothelial cell function have been examined across a range of time scales. Not unexpectedly, chronic glucose exposure induces long term tissue effects. Yet short term exposure can also impose lasting consequences. The persistence of vascular pathology after euglycemic restoration further suggests a glucose exposure memory. Slow turnover reservoirs such as basement membrane are candidates for prolongation of acute events. We hypothesized that glucose-induced vascular dysfunction is related to altered vasoactive compound handling within the endothelial cell-basement membrane co-regulatory unit. Endothelial cell basement membrane-associated fibroblast growth factor-2 increased linearly with culture glucose within days of elevated glucose exposure. Surprisingly, basement membrane fibroblast growth factor-2 binding kinetics remained unchanged. The glucose-induced increase in basement membrane fibroblast growth factor-2 was instead related to enhanced endothelial cell fibroblast growth factor-2 release and permeability. Cellular fibroblast growth factor-2 release occurred concomitant with apoptosis but was not blocked by caspase inhibitors. These data suggest that release was associated with sub-lethal early apoptotic cell membrane damage, perhaps related to reactive oxygen species formation. High glucose basement membrane in turn enhanced endothelial cell proliferation in a fibroblast growth factor-2-dependent manner. We now show that glucose-induced alterations in endothelial cell function promote changes in basement membrane composition, and these changes further effect endothelial cell function. These data highlight the interrelationship of cell and basement membrane in pathological conditions such as hyperglycemia. These phenomena may explain long term effects on the endothelium of short term exposure to glucose extremes.

Glucose is critical across the spectrum of biologic and physiologic processes. Yet in overabundance this sugar can produce profound systemic toxic effects. A primary manifestation of morbidity and mortality in diseases of glucose metabolism is unregulated remodeling and abnormal homeostasis of the vasculature. Much research has focused on long term exposure to elevated glucose, for example in diabetics. Indeed, though variable and vascular bed-dependent, vascular disease is diffuse and devastating in the diabetic and worse still with poor control of plasma glucose. Loss of renal filtration function in diabetic nephropathy follows glomerular capillary hypertrophy, and fragile new vessels formed by excessive angiogenesis create a unique retinopathy (1). Reduced angiogenesis in the extremities contributes to poor wound healing (2). In the macrovasculature, diabetics demonstrate accelerated atherosclerosis, rapid and more extensive restenosis after endovascular intervention, and decreased collateral formation around blockages (3, 4). These glucose effects persist long after restoration of euglycemia, and the possibility remains that acute fluctuations in local glucose concentration have prolonged cell and tissue effects.

Alterations in vascular basement membrane, a dense amorphous protein mesh located basolateral to endothelial cells, may explain hyperglycemic memory in diabetic vascular disease. Historically, vascular basement membrane was viewed purely from a structural perspective as a platform on which endothelial cells reside. More recently, it has become clear that basement membrane supports a plethora of biochemical activities, including cell signaling mediation through integrin binding, storage and release of growth factors such as vascular endothelial growth factor and fibroblast growth factor-2 (FGF-2)² and, even in degradation, production of protein fragments that promote or inhibit angiogenesis (5–9).

Diabetes changes basement membrane size and composition in both the micro- and macrovasculature. Throughout vascular beds, from kidney to eye to aorta, basement membrane thickens (1). Relative quantities of major protein components change, with increases in type IV collagen and fibronectin and decreases in laminin and heparan sulfate proteoglycans (1, 10–12). Histological analysis of human tissues has identified changes in basement membrane cytokine concentration, such as increased retinal FGF-2 in proliferative retinopathy (13). Although no single biochemical diabetic alteration has been identified as the cause of these changes, hyperglycemia has been implicated (14, 15). In vitro, high glucose affects basement membrane protein

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² The abbreviations used are: FGF-2, fibroblast growth factor-2; tBHP, tert-butyl hydroperoxide; TNFα, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; PECAM, platelet endothelial cell adhesion molecule; Ve-cadherin, vascular endothelial cadherin; NS, not significant; ANOVA, analysis of variance.
production and leads to glycation of these proteins, altering both matrix-matrix and matrix-cell interactions (16–18).

Using an in vitro hyperglycemic model, we investigated the influence of glucose on FGF-2 handling within the endothelial cell-basement membrane co-regulatory unit. Basement membrane FGF-2 levels were determined in low and high glucose culture, and basement membrane FGF-2 binding kinetics was measured. The effects of glucose-induced changes in endothelial cell FGF-2 release, permeability, and apoptosis on basement membrane FGF-2 storage were quantified, and in vitro results were correlated with in vivo data. Finally, we determined the effects of altered basement membrane FGF-2 on endothelial cell proliferation. We now show that basement membrane FGF-2 increases linearly with glucose exposure due to endothelial cell function alterations and that the change in basement membrane composition further affects endothelial cell function over an extended time scale.

EXPERIMENTAL PROCEDURES

Cell Culture—Porcine aortic endothelial cells were isolated from Yorkshire swine aortae by the collagenase dispersion method and maintained in low glucose Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin, and 2% glucose (Invitrogen) (19). Culture media was changed every 48 h, and cells were used between passages 4 and 9.

For high glucose media, d-glucose was added to supplemented low glucose Dulbecco’s modified Eagle’s medium (5 mM, 90 mg/dl) to a final concentration of 30 mM (540 mg/dl). Media glucose levels decreased over 4 days of culture due to cellular glucose consumption (5 mM glucose decreased to 2.4 ± 0.04 mM; 30 mM decreased to 22.4 ± 1.8 mM). Despite rapid endothelial cell glucose metabolism, the glucose level in high glucose media remained significantly higher than that of low glucose media over a 4-day experiment (p < 0.01). Although glucose effects continued to rise with higher glucose concentrations, values chosen are literature standards and remain close to the pathophysiologic range. For osmotic controls, mannitol (Sigma) was added to base media to achieve a 30 mM solution.

Porcine tumor necrosis factor-a (TNFα; R&D Systems), a potent inflammatory cytokine, and tert-buty lhydroperoxide (tBHP; Sigma), which is broken down intracellularly to produce reactive oxygen species, were used as alternative methods to induce cell stress. Endothelial cells were exposed to TNFα for 24 h and tBHP for 48 h. Recombinant human FGF-2 was obtained from Peprotech, and blocking antibodies for FGF-2 and TNFα were from Upstate Biotechnology and Serotec, respectively. The blocking antibodies specifically neutralized FGF-2 and TNFα when compared with a vascular endothelial growth factor antibody as measured through enzyme-linked immunosorbent assay (ELISA) and apoptosis assays.

Basement Membrane Isolation—Initial experiments demonstrated differential glucose effects on endothelial cells by 4 days; therefore, subsequent experiments used endothelial cells seeded near confluence and grown for 4 days in multiwell tissue culture polystyrene plates (12, 24, or 96 well; BD Biosciences). Cells in all glucose conditions reached confluence concurrently. 4% w/v dextran (40 kDa, Sigma) was added to media for the last 2 days to increase cellular basement membrane production (20). Endothelial cells were lysed in 20 mM NH4OH with 0.5% Triton X-100 (Sigma), after which wells were thoroughly washed with phosphate-buffered saline (6). Scanning electron microscopy confirmed that basement membrane remained intact, adherent to the tissue culture plate, and free of cell debris. The total basement membrane protein for each condition was quantified by the Coomassie assay after digesting isolated basement membrane with trypsin (25 μg/ml) for 24 h at 37 °C (21).

FGF-2 Extraction, Collection, and Quantification—Basement membrane FGF-2 was extracted using a salt buffer (2 mM NaCl, 20 mM HEPES, pH 7.4), which was deemed sufficient after subsequent extractions failed to produce additional FGF-2 (22). Total protein levels in FGF-2 extraction buffer ranged from 30 to 70 pg/ml and were not significantly different with glucose or osmotic controls. We further exposed isolated basement membrane to supplemented media for 4 days at 37 °C and were unable to extract measurable levels of FGF-2, confirming that exogenous FGF-2 from culture media fetal bovine serum did not significantly contribute to FGF-2 detected in basement membrane.

Endothelial cell-released FGF-2 was measured in media collected from cell culture and centrifuged to remove cellular debris. Tissue FGF-2 was prepared by homogenizing 3 × 3-mm porcine aortic tissue samples in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) with Complete protease inhibitor (Roche Applied Science). Homogenized samples were incubated on ice for 30 min and centrifuged to pellet insoluble material. All FGF-2 samples were either used immediately or stored at −20 °C for later use. Basement membrane, cell-released, and tissue FGF-2 levels were quantified using an FGF-2 ELISA (R&D Systems). Extraction buffer, conditioning media, or tissue lysate buffer alone did not alter ELISA accuracy. Because each ELISA varied slightly, as did FGF-2 integrity in various buffers, FGF-2 was quantified immediately after each experiment using a single ELISA kit whenever possible.

FGF-2 Binding Kinetics—All basement membrane FGF-2 binding kinetic experiments were performed at room temperature. Basement membrane FGF-2 equilibrium binding capacity was determined by adding FGF-2 in binding buffer (25 mM HEPES, 0.05% w/v gelatin, pH 7.4) to isolated basement membrane at concentrations from 0 to 1 μg/ml. Equilibrium, defined as the time at which association and dissociation occur at balanced rates resulting in no change in basement membrane-bound FGF-2, occurred 2–3 h after growth factor addition. The FGF-2 solution was aspirated, basement membrane was washed in binding buffer to remove unbound FGF-2, and bound FGF-2 was extracted as described above. Basement membrane FGF-2 association was measured by adding 5 ng/ml FGF-2 in binding buffer to isolated basement membrane for 0–360 min. This concentration (5 ng/ml) is within the linear binding range and results in physiologically relevant bound FGF-2 levels. After the incubation period, FGF-2 was aspirated, basement membrane was washed in binding buffer, and bound FGF-2 was extracted as previously described (22).
Glycated FGF-2 was prepared by incubating 1 μg/ml FGF-2 in 0.25 M fructose for 24 h at 37 °C (23).

FGF-2 basement membrane dissociation kinetics were determined by incubating isolated basement membrane with 5 ng/ml 125I-labeled FGF-2 in binding buffer to equilibrium (3 h). The 125I-labeled FGF-2 solution was removed, and binding buffer containing unlabeled FGF-2 (1 μg/ml) was added to each well for 0–360 min. Unlabeled FGF-2 was included in dissociation buffer to decrease rebinding of released 125I-labeled FGF-2 to the basement membrane. After the dissociation period, dissociation buffer was removed, and basement membrane-bound 125I-labeled FGF-2 was extracted (22). 125I-labeled FGF-2 in dissociation buffer and extraction buffer was quantified in a gamma counter (Packard).

Endothelial Cell Permeability—Endothelial cells were cultured for 4 days on 0.4-μm Costar Transwell inserts (Corning) in either 5 or 30 mM glucose supplemented media. After 4 days of glucose exposure, supplemented media was replaced by serum-free media with 1 mg/ml 10-kDa tetramethyl rhodamine-labeled dextran (Molecular Probes). At this size, the effective molecular radius of dextran resembles that of FGF-2. 50-μl samples were collected from media outside the insert well from 30 to 480 min after dextran addition and measured at 544/590 nm in a Fluoroskan fluorometer (ThermoLabsystems). Cell permeability to FGF-2 and subsequent binding to basement membrane was measured on cells cultured for 4 days in 5 or 30 mM glucose supplemented media. After 4 days of glucose exposure, supplemented media was replaced by serum-free media with 1 μg/ml FGF-2. After a 2-h incubation at 37 °C, basement membrane was isolated, and FGF-2 was extracted as described above.

Apoptosis and Cell Cycle Analysis—Endothelial cell apoptosis was measured using four complementary methods: cell counts, annexin V-propidium iodide labeling, caspase 3 activation, and terminal deoxynucleotidyltransferase dUTP nick end labeling. Cell counts were performed by trypsinizing cells and counting with a Coulter counter (Beckman Coulter). Annexin V binds phosphatidylserine translocated from the inner to the outer cell membrane. Cells in early apoptosis are identified as annexin V-positive and are negative for the vital dye propidium iodide. Endothelial cells were prepared for the annexin V-propidium iodide assay by combining floating and trypsin-released attached cells. Samples were centrifuged to pellet cells, washed thoroughly, resuspended in annexin binding buffer, and labeled with annexin V-fluorescein and propidium iodide as per the kit instructions (BD Pharmingen). Samples were analyzed immediately by flow cytometry (BD FACSan).

Caspase 3, a cysteine protease activated during early apoptosis, is considered the “point of no return” for apoptotic cells. Activated caspase 3 was measured in endothelial cells both by a spectrofluorometric assay and flow cytometry. For spectrofluorimetry, endothelial cells were lysed as per the kit instructions (BD Pharmingen), and 50 μl of cell lysate was incubated with 5 μl of N-acetyl-DEVD-7-arnino-4-methylcoumarin in a multiwell plate for 1 h at 37 °C. Ac-DEVD-aldehyde, a caspase inhibitor, was used as the negative control. 7-Amino-4-methylcoumarin (AMC) liberated from Ac-DEVD-AMC by activated caspase 3 was measured using a Fluoroskan at 380/450 nm. For flow cytometry, cells were collected as for the annexin V assay. Cells were fixed and permeabilized, then labeled with fluorescein-conjugated monoclonal rabbit anti-active caspase-3 antibody as per the kit instructions (BD Pharmingen). Samples were analyzed immediately by flow cytometry.

The terminal deoxynucleotidyltransferase dUTP nick end labeling assay employed an APO-BRDU kit (BD Pharmingen) in which TdT catalyzes the addition of bromomodal deoxyuridine triphosphates to 3’-OH ends of double- and single-stranded DNA fragments in late apoptosis. Cells were collected in the same way as for the annexin V assay. Cells were fixed in paraformaldehyde, washed, resuspended in 70% v/v ethanol in phosphate-buffered saline, and stored at −20 °C until use. For each experiment, samples were thawed, labeled as per kit instructions, and analyzed immediately by flow cytometry.

For each cell cycle analysis, endothelial cells were trypsinized, centrifuged, and resuspended at 37 °C in a stain solution of 3% w/v polyethylene glycol, 50 μg/ml propidium iodide (Molecular Probes), 180 units/ml RNase (Worthington), and 0.1% Triton X-100 in a 4 mM citrate buffer, pH 7.2. An equal volume of salt solution (3% w/v polyethylene glycol, 50 μg/ml propidium iodide, and 0.1% Triton X-100 in 0.4 M NaCl, pH 7.2) was added 20 min after 20 min. Cells were incubated overnight at 4 °C and measured by flow cytometry in the morning.

Fluorescence Microscopy and Immunohistochemistry—Endothelial cells cultured on coverslips were fixed in 4% v/v paraformaldehyde, pH 7.4. Fixed cells were labeled with mouse monoclonal antibody to platelet endothelial cell adhesion molecule (PECAM, CD31) (1:100, MCA1746, Serotec) and vascular endothelial cadherin (Ve-cadherin) (1:50, MCA1748, Serotec). After thorough washing, cells were labeled with Alexa Fluor 488 goat anti-mouse secondary antibody (1:100, A11017, Molecular Probes) and with Hoescht 33258 nuclear stain (1:1000). Coverslips were mounted on microscope slides with 1:1 glycerol-phosphate-buffered saline and imaged using either a 20×/0.5 or 40×/0.75 objective (Leica) in an inverted fluorescent microscope (CTRMIC; Leica). Hoescht and PECAM or Ve-cadherin images of the same microscope field were merged using Meta morph software, and background was subtracted uniformly from all images with Photoshop (Adobe). Intracellular and intercellular area were measured by tracing PECAM-labeled cell borders and counting pixels included or excluded, respectively, in Photoshop.

Immunohistochemical analysis was performed on frozen tissue sections (8 μm). Sections were acetone-fixed and air-dried for 24 h. Endogenous peroxidase activity was first quenched by incubating sections with peroxidase block, after which sections were incubated with mouse monoclonal antibody to FGF-2 (1:250, MC-GF1, Abcam). Mouse nonspecific IgG served as the negative control. Sections were incubated with a peroxidase-labeled dextran polymer conjugated to goat anti-mouse IgG (Dako Cytomation). Staining was completed by incubation with 3,3′-diaminobenzidine+ substrate-chromogen, which results in a brown-colored precipitate at FGF-2 sites. Slides were counterstained with hematoxylin and mounted using Crystal Mount Aqueous Mounting media (Sigma). Sections were imaged using a 60×/1.40 oil objective (Nikon) with a
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Nikon Labophot-2 bright field microscope. Images were analyzed using Photoshop.

Animal Model—Male domestic Yorkshire swine, initially 27–29 kg, were assigned to a control (n = 2) or diabetic group (n = 4). Before the study a vascular access port (Access Technologies) was surgically implanted into each pig via left external jugular vein catheterization (24). All pigs received intramuscular buprenorphine (0.03 mg/kg) on the day of surgery. Anesthesia was induced with intramuscular xylazine (2 mg/kg), atropine (0.04 mg/kg), butorphanol (0.55 mg/kg), and Telazol (6.6 mg/kg). Pigs were intubated, and anesthesia was maintained with isoflurane inhalant (0.5–1.5%) via an endotracheal tube.

Diabetes was induced by streptozotocin injection (50 mg/kg in 0.1 mol/liter sodium citrate, pH 4.5) each day for 3 days (25). All animals were fed a normal diet. Fasting blood glucose concentrations were measured once a week using a standard, portable glucometer. Blood glucose concentrations were maintained at 200–250 mg/dl by adjusting daily insulin injections for the 9-week study period duration (24). Serum lipids were measured at end point in a routine diagnostic analyzer using enzymatic colorimetric assays. Insulin therapy consisted of a mixture containing regular and NPH insulin (Eli Lilly). At the study completion, animals were anesthetized and euthanized with KCl, 40 mEq intravenous. An abdominal aortic segment was rapidly excised, washed thoroughly in phosphate-buffered saline, and frozen in liquid nitrogen. Aortic samples were stored at −80 °C until use.

This study conformed to United States Department of Agriculture regulations and National Research Council guidelines and to guidelines specified in the National Institutes of Health Guide for Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of Harvard Medical School (Boston) approved the study.

Statistical Analysis—All statistical analyses were performed with Prism software (Graphpad). Data were normally distributed and expressed as the mean ± S.D. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than 2 groups were analyzed by ANOVA. A value of p < 0.05 was considered statistically significant and is indicated in the text as such or in figures with a pound sign (#). A value of p < 0.01 is indicated with an asterisk (*).

RESULTS

Basement Membrane FGF-2—FGF-2, among other angiogenic growth factors, has been implicated in diabetic vascular dysfunction. Here, we show that the FGF-2 concentration in the endothelial cell basement membrane increases with both time and glucose exposure. After only 4 days in culture, basement membrane from cells cultured in 30 mM glucose stored significantly more FGF-2 than that from cells cultured in 5 mM glucose (p < 0.005). Only D-glucose increased basement membrane FGF-2; osmotic control mannitol was without effect on

![Figure 1A](http://www.jbc.org/figure1a.png)

**Figure 1A.** FGF-2 extracted from basement membrane increases with both time and cell culture glucose level. Porcine aortic endothelial cells were cultured for 2, 4, or 6 days in supplemented media of 5, 17.5, or 30 mM glucose. At the end of each time period, cells were removed, and FGF-2 was extracted from basement membrane and measured via FGF ELISA. p < 0.0001 (ANOVA); *p < 0.01 among 5, 17.5, and 30 mM glucose for marked time point. Shown are the mean ± S.D., n = 3.

![Figure 1B](http://www.jbc.org/figure1b.png)

**Figure 1B.** Basement membrane FGF-2 does not increase with mannitol osmotic controls. Porcine aortic endothelial cells were cultured for 4 days in supplemented media with 5, 17.5, or 30 mM D-glucose or mannitol. Cells were removed, and FGF-2 extracted from basement membrane and measured via FGF ELISA. *p < 0.01 when compared with 5 mM glucose. Shown are the mean ± S.D., n = 3. C, basement membrane FGF-2 increases linearly with endothelial cell culture glucose. Porcine aortic endothelial cells were cultured for 4 days in supplemented media with glucose ranging from 5 to 50 mM. After 4 days cells were removed, and FGF-2 extracted from basement membrane and measured via FGF ELISA. p < 0.0001, r² = 0.79 (ANOVA). Shown are the mean ± S.D., n = 3.
basement membrane FGF-2 levels (Fig. 1B). Basement membrane FGF-2 increased linearly up to 50 mM culture glucose (Fig. 1C, \( r^2 = 0.79 \)). TNFα and tBHP addition resulted in similar dose-dependent increases in FGF-2, but neutralization of TNFα did not abrogate the glucose effect on basement membrane FGF-2 (Table 1).

**Basement Membrane-FGF Binding Kinetics—** We examined basement membrane FGF-2 binding kinetics to elucidate the role of glucose-induced basement membrane alterations in increasing FGF-2 storage. No significant difference in basement membrane FGF-2 binding capacity with culture glucose was observed across a wide range of FGF-2 concentrations (Fig. 2A). Equilibrium binding capacity remained linear far beyond the physiologic range (0–100 ng/ml; \( p < 0.0001 \), \( r^2 = 0.99 \)), reaching a plateau between 5 and 10 µg/ml soluble FGF-2. Similarly, both FGF-2 association and dissociation with isolated basement membrane were unchanged by glucose whose basement membrane was grown in varied glucose (association data Fig. 2B; dissociation data not shown) or glucose was present in binding buffer. Only FGF-2 glycation significantly altered basement membrane FGF-2 binding kinetics, leading to decreased association with basement membrane (Fig. 2B). These data were confirmed by measuring FGF-2 binding to a heparin-Sepharose column. These data then suggest that the increase in basement membrane FGF-2 with glucose is not related to an inherent change in binding kinetics.

**Basement Membrane Exposure to FGF-2—** We next considered whether basement membrane was exposed to more soluble FGF-2 through glucose-induced endothelial cell FGF-2 release. With a rise in glucose concentration from 5 to 30 mM, endothelial cells released twice as much FGF-2 into the media over a 48-h period (Fig. 3). A similar dose-dependent increase in media FGF-2 was observed when TNFα or tBHP was added to culture media but not with osmotic control mannitol.

Endothelial cell permeability with glucose was investigated to determine whether released FGF-2 could access and bind to basement membrane. Because FGF-2 did not traverse the Transwell insert membrane without some binding, dimensionally equivalent but inert 10-kDa dextran was examined. Cell permeability to dextran after 4 days of glucose exposure increased acutely in a glucose dose-dependent manner when new glucose media was added (Fig. 4A). Cell permeability peaked at ~90 min and returned to baseline conditions by 180 min for 17.5 mM glucose and 360 min for 30 mM glucose. Specific endothelial cell permeability to FGF-2, measured by extracting FGF-2 in basement membrane after applying an FGF-2 load, also rose with exposure to glucose and low levels of TNFα or tBHP (Fig. 4B). FGF-2 bound to basement membrane increased 6-fold in cells exposed to TNFα or tBHP over unexposed cells.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Environmental stress</th>
<th>Glucose (mM)</th>
<th>TNFα (ng/ml)</th>
<th>tBHP (mM)</th>
<th>Glucose + TNFα antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>15.6 ± 1.2</td>
<td>17.9 ± 2.6</td>
<td>13.6 ± 0.7</td>
<td>16.5 ± 1.7</td>
</tr>
<tr>
<td>Medium</td>
<td>23.6 ± 4.3</td>
<td>25.4 ± 2.4</td>
<td>20.3 ± 2.5</td>
<td>27.6 ± 5.9</td>
</tr>
<tr>
<td>High</td>
<td>30.0 ± 3.6</td>
<td>41.3 ± 6.4</td>
<td>59.3 ± 4.6</td>
<td>32.1 ± 3.9</td>
</tr>
</tbody>
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* \( p < 0.05 \)

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Continuous PECAM and Ve-cadherin along cell borders for all glucose levels (Fig. 5) indicated that the increase in endothelial cell permeability occurred without significant changes in cell-cell adherens junctions. Most interesting was the increase in two-dimensional cytoplasmic area (3260 ± 153 to 5033 ± 310 μm², p < 0.0001), as well as a 2-fold increase in intercellular area with glucose concentration. A similar increase in cytoplasmic area, but not intercellular area, was observed in osmotic controls.

**Endothelial Cell Apoptosis—Glucose, TNFα, and tBHP** each demonstrated a dose-dependent increase in early apoptotic cells, as measured by the annexin V-propidium iodide assay (Fig. 6). We confirmed by caspase-3 activation, terminal deoxynucleotidyltransferase dUTP nick end labeling flow cytometry, and cell counts that a higher percentage of cells underwent apoptosis with each agent but not mannitol (data not shown). Yet while a general caspase inhibitor decreased caspase-3 positive cells by more than 50% in high glucose conditions (p < .001), it did not significantly affect the percentage of annexin V-positive propidium iodide-negative cells (4.16 ± 0.27 versus 4.14 ± 0.17, p = NS). The caspase inhibitor also did not reduce basement membrane FGF-2 for the 30 mM glucose endothelial cells (89.6 ± 2.5 pg/cm² with inhibitor, 77.3 ± 8.5 pg/cm² with inhibitor, p = NS) to the 5 mM glucose endothelial cell level (39.7 ± 2.7 pg/cm² with inhibitor, 39.0 ± 1.8 pg/cm² with inhibitor, p = NS).

**Basement Membrane FGF-2 in Vivo—FGF-2** levels were measured in aortae from control and streptozotocin-treated pigs to validate that the increase in basement membrane FGF-2 was not restricted to endothelial cells in culture but was a real and valid effect in intact vessels in vivo. On average, fasting blood glucose of pigs treated with streptozotocin was 4-fold higher (267 ± 100 mg/dl) than in control animals (68 ± 4 mg/dl, p = 0.02). Diabetic pigs also had higher plasma triglycerides than controls (21 ± 2 versus 17 ± 1, p < 0.01) and a non-significant decrease in body weight (65 ± 9.6 versus 74.8 ± 0.8, p = NS). Immunohistochemical FGF-2 analysis of porcine aortic sections showed greater FGF-2 throughout the arterial wall in hyperglycemic animals (Fig. 7A). The increase was most pronounced in the subendothelial layer. When FGF-2 was measured in tissue extracts, FGF-2 normalized to total protein extracted was 44% higher in diabetic animal tissue (Fig. 7B).

**Endothelial Cell Growth on the Basement Membrane—** After 7 days in low glucose growth media, viable endothelial cell number was 25% higher on either 30 mM glucose basement membrane or 5 mM glucose basement membrane with added FGF-2 as compared with 5 mM glucose basement membrane
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Endothelial cells and basement membrane operate as an integrated co-regulatory unit. Cells produce basement membrane proteins, and basement membrane contributes to subsequent cell-signaling regulation (26). Vlodavsky et al. (6) expanded the bidirectional influence of cells and basement membrane by showing that bioactive cell-derived proteins such as FGF-2 are stored in basement membrane for later release. We now show that basement membrane contributes a memory component to bioregulation and vascular homeostasis. Environmental stress in disease, such as hyperglycemia in diabetes, modifies basement membrane protein balance via short term alterations in endothelial cell function. These acute changes then exert long term effects on endothelial cell function.

Our work is novel in that we demonstrate a link between glucose-induced endothelial cell dysfunction and basement membrane FGF-2 changes. Hyperglycemia has long been known to alter both endothelial cells and basement membrane as well as endothelial cell production of basement membrane proteins (12, 15, 27). However, we are the first to link hyperglycemic changes in endothelial cell function, in particular apoptosis and permeability, to an alteration in basement membrane cytokine storage. Our research further demonstrates for the first time how increased basement membrane FGF-2 can specifically alter endothelial cell function, highlighting the interrelationship of cell and basement membrane changes in diabetes. Thus, the novel nature of our work elucidates glucose-induced dysfunction within the endothelial cell-basement membrane FGF-2 feedback loop.
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The short term deleterious effects of hyperglycemia, such as alterations in plasma concentrations or pH, are well recognized as clinical syndromes like hyperosmolar coma and diabetic ketoacidosis. Similarly, advanced glycation end products have recently come into question. Cowan et al. (30) showed that caspases are released during apoptosis, and these released caspases degrade elastase in the surrounding environment. Because FGF-2 promotes endothelial cell survival and proliferation, FGF-2 release early in apoptosis could be critical in limiting cell death and promoting regrowth both during initial injury and over time through FGF-2 basement membrane storage.

FGF-2 release was not blocked by caspase inhibition, suggesting that release occurs either before caspase activation or despite caspase inactivation. FGF-2 release timing was consistently associated with annexin V labeling, an indicator of early apoptotic cell membrane damage. FGF-2 release from endothelial cells could occur during early apoptotic loss of membrane integrity and, therefore, would not be prevented with caspase inhibitors that block apoptosis at a later stage (31). Alternatively, cell death similar to apoptosis but without caspase activation or despite caspase inhibition has recently been proposed (32). Endothelial cells exposed to high glucose may continue to die through caspase-independent pathways, resulting in FGF-2 release.

Whereas high glucose is known to increase endothelial cell permeability to inert proteins such as albumin in vitro, we now show that glucose enhances permeability to a biologically active factor (27). Enhanced permeability in atherosclerosis-prone areas in vivo is linked to arterial albumin, fibrinogen, and low density lipoprotein cholesterol deposition. Our results show for diabetic complications. The biological effects detailed in this paper, however, occur across time scales that had previously been studied separately. Our experiments demonstrate how glucose fluctuations on the order of days lead to basement membrane alterations, which may account for lasting vascular effects.

The increased basement membrane FGF-2 storage demonstrated here in vitro may be even more extensive in vivo. In our in vitro experiments, basement membrane achieved a constant protein level by 4 days, indicating that further change in basement membrane FGF-2 after this time was related to FGF-2 exposure rather than additional basement membrane production. In vivo, increased basement membrane FGF-2 exposure with time is coupled with a documented increase in vascular basement membrane thickness over years of diabetes. Basement membrane FGF-2 could rise even further as the number of total binding sites increases. One such example is the colocalized increase in basement membrane thickness and FGF-2 in diabetic proliferative retinopathy (13). Even sites far from endothelial cells could store growth factor, as FGF-2 can diffuse through the vessel wall with rapid reversible binding (28). In our studies, this effect is evidenced by high FGF-2 levels throughout the aortic media of diabetic porcine tissue (Fig. 7A).

Our data suggest that FGF-2 is released from endothelial cells through sub-lethal membrane injury early in the apoptotic process. With no known signal sequence for secretion, FGF-2 is postulated to be released only at cell injury or death (29). Yet apoptosis has classically been considered as clean cell death with little to no cellular release. This is in direct contrast to necrosis, where cell death causes cellular content spillage and affects surrounding cells. However, this apoptotic paradigm has recently come into question. Cowan et al. (30) showed that caspases are released during apoptosis, and these released caspases degrade elastase in the surrounding environment. Because FGF-2 promotes endothelial cell survival and proliferation, FGF-2 release early in apoptosis could be critical in limiting cell death and promoting regrowth both during initial injury and over time through FGF-2 basement membrane storage.
the first time that glucose-induced permeability alters basement membrane and vascular wall FGF-2 content (33). Glucose-induced cell permeability has been linked to protein kinase C activation and vascular endothelial growth factor (27). Although the specific protein kinase C inhibitor bisindolylmaleimide and a neutralizing vascular endothelial growth factor antibody effectively inactivated their targets, both were ineffec-
tive in blocking glucose-induced permeability in our experi-
ments (data not shown). Instead, the permeability increase could occur with early apoptotic changes or alternative forms of cell death, as it was seen across the chemical stressors but was not affected by caspase inhibition. Independent of biochemical mediation, glucose-induced permeability is likely related to loss of intercellular junction integrity. Unchanged VE-cadherin levels in conjunction with increased intercellular area (also observed by others through transmission electron microscopy) imply that tight rather than adherens junctions are affected (34–36). The intracellular area change (Fig. 5), which had been attributed to increased cellular metabolic function in high glu-
cose, was also observed with osmotic controls and, therefore, is expected to be nonspecific to glucose (37).

We hypothesize that glucose-induced changes in apoptosis, permeability, and basement membrane FGF-2 are related to intracellular reactive oxygen species formation. Reactive oxygen species have been suggested by others to be common medi-
ators for hyperglycemic cell damage (38). Glucose, TNFα, and tBHP all function via formation of intracellular reactive oxygen species, and the short time scale of glucose effects in our model correlates well with short term reactive oxygen species formation rather than long term glycation. In addition, Nishikawa et al. (39) suggested that mitochondrial superoxide production unifies three previously considered independent glucose biochemical pathways: protein kinase C activation, advanced gly-
cation end products, and sorbitol accumulation. Reactive oxygen species study is complicated by their delicate intracellular balance, with small quantities critical for intracellular signaling but large quantities severely impairing cell viability. In our experiments, superoxide dismutases decreased intracellular reactive oxygen species but also so inhibited vital cell functions that long term experiments were not possible. Therefore, fur-
ther research will be necessary to define the role of reactive oxygen species in hyperglycemic dysfunction within the endo-
thelial cell-basement membrane feedback loop.

Although neutralization of TNFα did not mitigate glucose effects on basement membrane FGF-2, the coupled increase in cellular bioactive factor release and permeability with TNFα is of interest in itself. The interleukins and interferon-γ also bind basement membrane, and recent studies have shown that base-
ment membrane binding of these factors increases their half-
life and local concentration (40). High TNFα levels in inflam-
mation could result in interleukin and interferon-γ deposition in the vascular wall where they may contribute to inflammatory process extension.

Unchanged FGF-2 basement membrane binding kinetics with glucose are surprising. There are extensive alterations in composition and structure of diabetic vascular basement mem-
brane, in particular decreased heparan sulfate proteoglycan content (12). Vogl-Willis et al. (41) measured small decreases in sulfation of basement membrane heparan sulfate proteoglycans in short term hyperglycemic endothelial cell culture. We simi-
larly observed a decrease in heparan sulfate proteoglycan sulfu-
tion with glucose but without effect on FGF-2 binding kinetics. Basement membrane binding kinetics were altered only if heparan sulfate proteoglycan content was drastically reduced, as in our hands and by others through sodium chloride in cul-
ture (21). Whereas glycation has been shown to decrease FGF-2 activity, heparan sulfate protects FGF-2 from glycation. This implies that the increased basement membrane FGF-2 store should remain unglycated and, therefore, active even over long hyperglycemic periods (23, 42).

Our in vivo data validate in vitro findings, and yet the model we employ is not without limitations. Animals were exposed to periodic exogenous insulin injections and developed profound metabolic changes in addition to hyperglycemia including increased triglycerides and decreased end point weight. Our immunohistochemical and ELISA extractions examine whole tissue sections rather than isolated basement membrane as in our in vitro studies. Nonetheless, in vivo data do extend in vitro findings by suggesting that long term hyperglycemia can lead to increased vascular FGF-2 storage.

Glucose modulates FGF-2 release from, and permeability between endothelial cells. Growth factor, stored in increased amounts in the hyperglycemic basement membrane, is available to act on vascular cells (endothelial, smooth muscle, and even resident inflammatory cells), which themselves are under hyperglycemic stress. The implications of these data are potentially far-reaching. We have shown definitively that endothelial cell function controls vasoactive factor storage in vascular base-
membrane and that these factors in turn specifically affect cell function. Endothelial cell permeability affects not only what diffuses from blood to tissue but what is retained within the vascular wall and affects the wall itself. Such autocrine/para-
crine control mechanisms, in particular the memory aspect of basement membrane storage, may explain why many diabetic effects last long after euglycemia has been restored. Our data further support shifting paradigms for apoptosis. Not only does cell death still occur with caspase inhibition, but FGF-2 release occurs whether death is caspase-dependent or -independent.

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